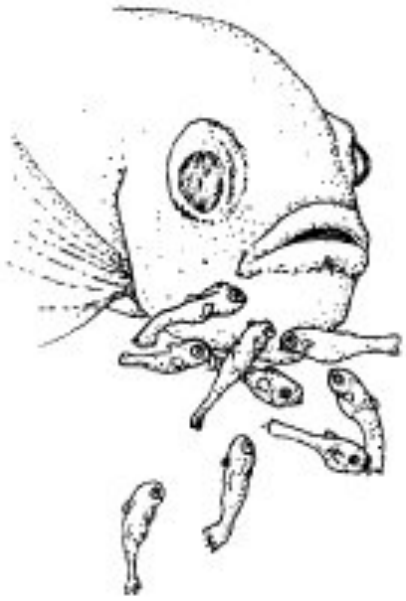
Parental investment and sexual immune dimorphism in cichlids and syngnathids



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FÜR FLORI UND MEINE ELTERN

"*In the context of parental care everything interacts with everything else*"
Huston et al. 2013

Contents

SUMMARY

Males and females differ in their reproductive investment, males produce small, but ample sperm and females invest in expensive eggs. This anisogamy leads to sexual dimorphism through a sex-specific resource allocation trade-off where females invest in life history traits to ensure longevity, while males invest in secondary sexual signals to ensure matings. In sex-role reversed species, females are limited in their reproductive success by males, inverting this selection gradient. Sex-specific resource allocation also affects the immune system, generating sexual immune dimorphism. Where, in conventional sex roles, the male often is immune suppressed compared to the female, and *vice versa* in sex-role reversed species.

Post-zygotic parental investment, perceived as parental care, might additionally influence sexual selection. Parental care itself is costly for the caregiving parent, both regarding transient provisioning costs as well as evolutionary costs, lowering the possibility for additional matings. Adaptations to parental care can thus enhance the already existing differences in sexual selection between the caregiving and the non-caregiving individuum. Provisioning costs, arising due to a resource allocation trade-off between parental care and the immune system, are only imposed upon the caregiving sex in times of brooding. Parental care patterns might influence the sex-specific allocation of resources and with this also sexual immune dimorphism.

In Chapter I of my thesis, I compared the interaction between parental investment and sexual immune dimorphism in maternal and biparental mouthbrooding cichlids. To test if sex-specific investment in parental care is more similar in biparental brooding species than in maternal brooding species, in both sexual immune dimorphism as well as evolutionary costs of parental care.

In Chapter II, I compared sexual immune dimorphism of three sex-role reversed syngnathid species during male pregnancy. The evolution of male pregnancy needs specific adaptations and involves high provisioning costs. By comparing males of different reproductive stages within and between species, I was able to assess both evolutionary and provisioning costs of male pregnancy and to investigate the influence of sex-role reversal and paternal investment intensity on sexual dimorphism in immune functions.

In Chapter III, I focused on a maternal mouthbrooding cichlid to assess the provisioning costs of reproduction and mouthbrooding in naïve versus immune-challenged females. The immune challenge increases the resource allocation trade-off between parental investment and the immune system allowing further conclusions about the provisioning costs of parental investment. This immune challenge also permitted investigating the existence and specificity of maternal immune priming of the offspring through the buccal mucosa. To this end,

juveniles of challenged and naïve females where mouthbred or artificially reared before exposing them to immune challenges homologous or heterologous to the maternal challenge.
My results suggest a dependence of sexual immune dimorphism on the parental care pattern and the mating system. Provisioning costs and evolutionary costs of intense parental investment are parental care mode specific and are traded off with the immune system. Further, they indicate that maternal stress negatively affects the juvenile physiological condition and the development of the immune system. In conclusion, my data revealed co-evolutionary dynamics among parental investment and the immune system with the aim to enhance reproductive success and as such maximise Darwinian fitness.

ZUSAMMENFASSUNG

Männchen und Weibchen unterscheiden sich in ihrer Investition in die Fortpflanzung. Während Männchen eine große Zahl von Spermien produzieren, tragen Weibchen größere und teurere Eier zur Reproduktion bei. Diese Anisogamy führt unweigerlich zu einer geschlechterspezifischen Ressourcenverteilung. Unter welcher Weibchen in ihre Langlebigkeit und Männchen in die Ausprägung sekundärer Geschlechtsmerkmale investieren, um ihren jeweiligen Reproduktiven Erfolg zu maximieren. Hieraus resultiert der sexuelle Dimorphismus. In Arten mit vertauschten Geschlechterrollen ist das Weibchen in ihrem Fortpflanzungserfolg durch das Männchen limitiert, wodurch sich der Selektionsgradient umkehrt. Geschlechterspezifische Investitionen sind ebenfalls im Immunsystem sichtbar. Im Vergleich zum Weibchen ist das Männchen in konventionellen Geschlechterrollen immunsupprimiert, auch dies kehrt sich in Arten mit vertauschten Geschlechterrollen um.

Postzygotische elterliche Investitionen, elterliche Pflege, kann den Selektionsgradienten zusätzlich beeinflussen. Der Mangel an Verpaarungsmöglichkeiten des pflegenden Elternteils erhöht den geschlechterspezifischen Unterschied und verstärkt somit den Selektionsgradienten. Zusätzlich ist elterliche Pflege ressourcenaufwendig, sowohl in adaptiven Merkmalen wie auch in vorübergehenden Kosten während der Pflege. Beide dieser Kosten führen zu einem Ressourcenverteilungsproblem zwischen der elterlichen Pflege und, unter anderem, dem Immunsystem. Elterliche Pflege kann also nicht nur den Selektionsgradienten beeinflussen sondern möglicherweise auch die geschlechterspezifische Verteilung von Ressourcen und damit den vorhandenen Sexuellen Immundimorphismus verstärken

Das erste Kapitel dieser Arbeit befasst sich mit der Interaktion zwischen elterlicher Investition und sexuellem Immundimorphismuns in maulbrütenden Buntbarschen mit maternalen oder biparentaler Pflege. Um herauszufinden ob in biparental brütenden Tieren die Ressourcen egalitärer von den Geschlechtern aufgebracht und somit beides, die aufgewendeten Anpassungskosten an solche Brutpflege Strategien aber auch der sexuelle Immunedimorphismus verglichen mit maternal brütenden Tieren, abgeschwächt sind.

Das zweite Kapitel untersucht inwiefern vertauschte Geschlechterrollen und intensive männliche Schwangerschaft in Seenadeln den sexuellen Immundimorphismuns und die evolutionären und vorübergehenden Kosten, beeinflusst. Die Evolution männlicher Schwangerschaft bringt viele spezifische, kostspielige Anpassungen und hohe vorübergehenden Kosten für die Männchen. Durch den Vergleich verschiedener Stadien in der männlichen Reproduktion innerhalb der Arten konnte ich die vorübergehenden Kosten der männlichen Schwangerschaft untersuchen. Die Kosten artspezifischer Adaptionen an verschiedene Ausprägungen dieser speziellen Fortpflanzungsart ergaben sich aus dem Vergleich der Arten untereinander. Durch den Vergleich der Weibchen und

Männchen der jeweiligen Art konnte ich den Einfluss verschiedener Ausprägungsarten von männlicher Schwangerschaft auf den sexuellen Immundimorphismus abschätzen.

Das dritten Kapitel meiner Arbeit konzentriert sich auf eine Art von maternal maulbrütenden Buntbarschen um die vorübergehenden Kosten von Reproduktion und Brüten in geimpften und naiven *Astatotilapia burtoni*-Weibchen abschätzen zu können. Das Impfen verschiebt die Ressourcenverteilung zwischen elterlicher Pflege und dem Immunsystem. Hieraus ergibt sich eine zusätzliche Ressourcenverknappung, welche die vorübergehenden Kosten elterlicher Pflege besser abschätzen lassen. Die Impfung ermöglicht ebenfalls den mütterlichen Einfluss auf das Immunsystem der Nachkommen zu untersuchen, da durch den engen Kontakt mit der mütterlichen Mundschleimhaut die Übertragung von Immunkomponenten möglich sein könnte. Dafür wurden die Nachkommen entweder von der Mutter ausgebrütet oder außerhalb der Mutter aufgezogen. Anschließend wurden die Juvenilen mit dem gleichen oder einem anderen Bakterium geimpft wie die Mutter und die Existenz und Spezifität eines möglichen maternalen Effektes zu messen.

Meine Resultate zeigen eine Abhängigkeit von sexuellem Immundimorphismus und elterlicher Pflege. Ein Einfluss des Paarungsverhaltens konnte außerdem aufgezeigt werden. Die vorübergehenden und die evolutionär bedingten Kosten elterlicher Investition scheinen abhängig von der jeweiligen Brutpflege zu sein führen aber immer zu einem Ressourcenverteilungskonflikt mit dem Immunsystem. Meine Ergebnisse deuten auf eine negative Beeinflussung der Kondition und der Entwicklung des Immunsystems der Nachkommen durch mütterlichen Stress während des Brütens hin. Zusammengefasst zeigen meine Daten eine koevolutionäre Dynamik zwischen dem sexuellem Immundimorphismus und der Brutpflege.

INTRODUCTION

1. Parental investment

According to Trivers parental investment is defined as "any investment by the parents in an individual offspring that increases the offspring's chance of surviving, as well as the parental reproductive success at the cost of the parent's ability to invest in future offspring" [1]. With this definition, Trivers induced a discussion about the relationship between past and future investment in parental care [2]. Several theoretical models with the aim to examine the origin and sex-specificity of parental investment and their influence on other life-history traits followed [3–5].

When assessing the evolutionary roots of parental investment, one should disentangle initial (i.e. provision of gametes), internal (i.e. incubation of eggs or embryos; viviparity or ovoviviparity), and external parental investment (i.e. parental care in a broader sense).

The initial investment, leading to the fusion of the energy- and nutrient-rich egg and the low energy sperm, is the fundament of reproduction [6]. While it has been assumed that additional energy is packed in large eggs, sperm was supposed to not contribute more than just the genomic DNA to the offspring. Recent data, however, suggest that the paternal contribution can be much larger via epigenetic modulation of offspring gene expression [7].

Internal investment requires physiological and morphological adaptations of the reproductive tract, which evolved several times independently in mammals, reptiles, and fish [8]. The evolution of both viviparity and ovoviviparity boosts offspring survival. At the same time, viviparity and ovoviviparity increase the costs for the parent(s) resulting in reduced fecundity and motility, as well as higher metabolism [9,10]. External parental investment, perceived as parental care, summarises all components of parental behaviour which are enhancing the offspring's fitness [6]. Such behavioural traits include territorial defence, nest building, defence against predators, oxygenation of embryo and acquisition of nutrients [11].

All aspects of parental investment entail a resource allocation trade-off between energy allocated to the offspring (gamete production, pregnancy, brooding and parental care) and the somatic maintenance, but also the survival of the parent [11]. Such *provisioning costs* are plastic and adjustable depending on the environment and the parental condition. Consequences of provisioning costs are a higher risk of predation, infection, injury and reproductive parasitism as well as time and energy loss for the caregiving parent [12–14]. Furthermore, internal and external care increase energy expenditure in the form of elevated metabolic rates and higher oxygen consumption [15,16], potentially leading to physiological, oxidative and glucocorticoid stress [17–20], depletion of energy stores and micronutrients, and immunosuppression [21,22]. In mammals, immunosuppression is especially high during gestation and implantation as a consequence of provisioning costs during these stages [23].

In addition to the provisioning costs, *evolutionary costs* of parental investment can be substantial. Such evolutionary costs are directly related to

Darwinian fitness and often result from life-history shifts and body adaptations to parental care [9,24,25]. Sex-specific life history strategies have co-evolved with parental investment, to this end, evolutionary costs can determine the sex roles and reproductive biology of a species. The importance of the evolution of parental investment is underlined by the unique diversity of parental care patterns and mating systems that evolved independently in the animal kingdom [26,27]. The evolution of parental investment truly represents one of the most spectacular examples of convergent evolution [28]. Evolutionary costs arise over conflicts about resources allocated to parental care both between parents and offspring as well as between sexes [29]. Furthermore, the caregiving parent has the costs of morphological and physiological body adaptation. An outstanding example is the suppression of the maternal immune response to promote maternal tolerance to the embryo during mammalian pregnancy [23].

2. Initial parental investment, anisogamy and sexual selection

Sexual conflict over the provisioning of parental care is inevitably bound to anisogamy, as differentially sized gametes are the initial and often sole sex-specific investment into the future offspring. Anisogamy defines females as producing few nutrient-rich eggs, whereas males produce small but many sperms. As sperm is cheap and abundant, male lifetime reproductive success (LRS) depends on how many eggs are fertilised (involved in this definition is the investment into secondary sexual signals). In contrast, eggs are scares and costly, and female LRS may thus be maximised by elongating the lifetime (Bateman´s principle) [30,31].

LRS of females and males are thus inherently different, which induces sex-specific selection and sex-dependent evolutionary strategies [1,6,30,32]. The larger the sex-specific differences in LRS, the stronger sexual selection may act on the sex limited in reproductive success, usually the male [33,34]. Sexual selection entails intrasexual selection or mate competition (the competition within one sex for access to the other) and intersexual selection or mate choice (selecting for traits in one sex favoured by the other sex). Males are mostly subject to both intra- and intersexual selection, as females are the limiting sex due to their higher initial investment into offspring and in some cases additional post-zygotic care [1,30,35].

In sex-role-reversed animals Bateman's principle is inverted: males have a lower potential reproductive rate through a greater post-zygotic parental care and represent the limiting resource for females being under stronger sexual selection.

3. Sexual immune dimorphism

Sexual selection led to the evolution of secondary sexual traits displayed as honest signals to increase mating success of the sex limited in its reproductive success[36]. This sex-specific trait expression entails morphological differences in coloration and size (whereby the male is often more colourful and bigger than the female) but also includes differences in physiology, behaviour and gene

expression [37]. Honest secondary sexual traits represent the quality and health of the male [38]. Their display is heritable and costly [36,38,39]. As such, the display of secondary sexual signals was suggested to correlate with higher parasite resistance and the provisioning of good genes for the offspring [36,40]. Only high-quality males can allocate their resources towards secondary sexual signals due to a resource allocation trade-off between ornamentation and all other life-history traits [41]. By choosing highly ornamented males, the female ensures sperm of good quality for her offspring [36,41,42].

The expression of secondary sexual signals is boosted by high androgen levels that may have immune suppressive consequences [41,42]. This correlation was used as an explanation for intensified infections and more severe symptoms on the male side [43]. Such sexual immune dimorphisms have been identified in various species 36,41,44,45.

Mediation of sexual immune dimorphism through androgens came into question, when sexual immune dimorphism was also found in invertebrates lacking these sex hormones[46,47]. In addition, sex-role reversed males also have higher levels of androgens but showing a higher immune competence than females [48–50]. Rolff (2002) revolutionised the field of *sexual immune dimorphism* by applying Bateman's principle to the immune system, which gives an explanation without allocating a function to hormones. As mentioned above, according to Bateman, male are limited by access to females, whereas females are limited by the numbers of eggs produced [30]. As females have a higher time investment per reproductive unit, they need to elongate their life span to maximise their reproductive success. Accordingly, for females, an efficient immune response to prevent pathogen and parasite infection is vital to increase longevity, while males rather allocate their resources in intersexual selection resulting in a lower immunocompetence [44]. Consequently, the distinct sexual selection between males and females might explain the difference in immune competence observed in both vertebrates and invertebrates.

Correspondingly, sexual immune dimorphism is suggested to be more pronounced in species with strong intersexual selection [51], as more resources are allocated towards sexual conflicts to gain matings [45]. In species with a similarly strong sexual selection and reproductive investment in males and females, sexual immune dimorphism is proposed to be less distinct [43,52].

As also parental investment is influenced by sexual selection, an interrelation between sexual immune dimorphism and extent of parental investment is expected.

4. Evolution of post-zygotic parental care:

Clutton Brook (1991) suggested extending Bateman´s principle by including parental care, as a factor to increase the rate of offspring production. This entails that post-zygotic parental care, rather than gamete production, is potentially limiting lifetime reproductive success. Thus, the sex providing post-zygotic care becomes the limiting sex, and the other is subjected to stronger sexual selection [6].

As zygote survival is induced by parental care, a reduction in care by one parent has to be compensated by the other parent [53]. Unless care of both parents is needed for optimal offspring survival, this creates sexual conflict over parental care, as both parents aim to elevate their LRS [54]. The higher the uncertainty of parentage, the lower the fitness benefits gained by caring for the offspring. Hence, males often desert if paternity is unsure due to extra pair copulations [4]. In general, species with below average paternity per mating often show reduced parental care [55]. A prominent exception from this rule is the prevalent male care observed in fish. Through territoriality, where many females lay their eggs in the male territory and desert, males are more secure about parentage and care for the egg desert [56,57].

Additionally, the operational sex ratio (OSR) might explain the evolution of sex-specific parental care strategies [5]. The OSR is the ratio between adult males and females available for reproduction and not engaged in brood care. The sex being rarer within a population will gain less from providing care, as it easily finds new mates, and will thus rather choose to desert and remate [1,2]. The effect on the OSR on differential parental care pattern can be observed in the St. Peter's Fish. Parental care in this species is plastic and dependent on the OSR either of the three: maternal, paternal or biparental [58].

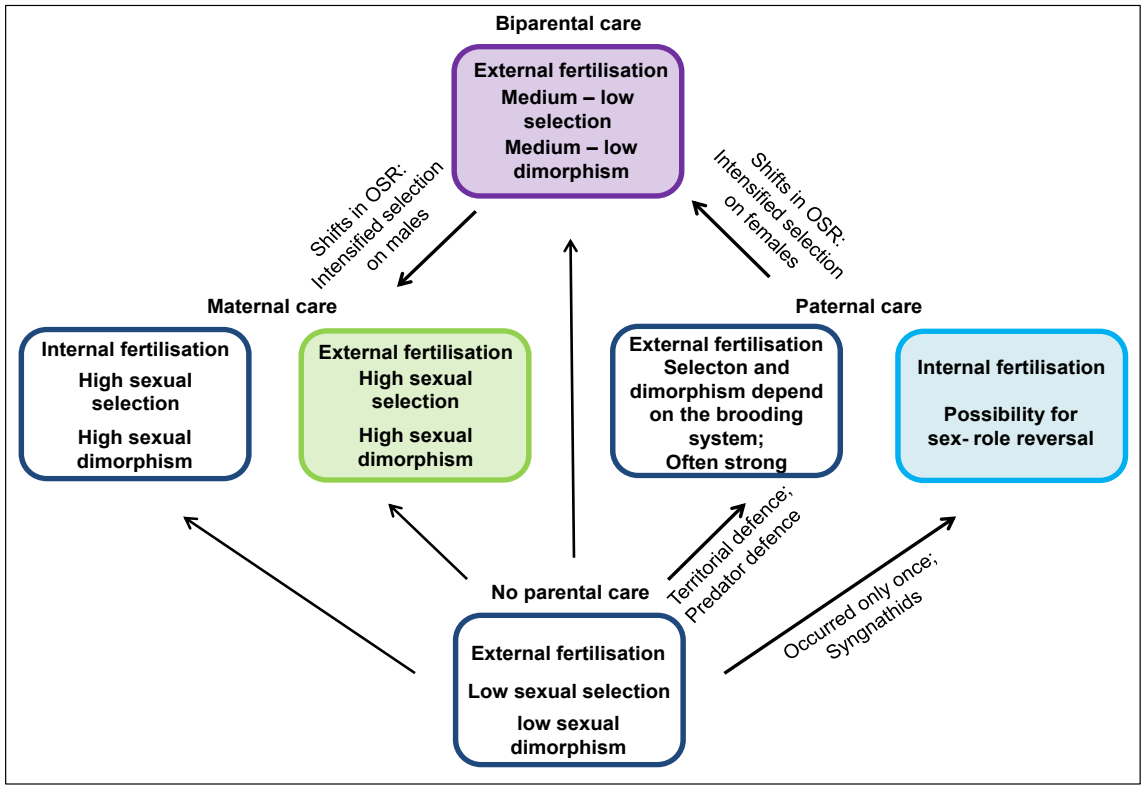

Figure 1: Schematic overview on the transitions of parental care in teleost. Interaction between sexual dimorphism, sexual selection, fertilisation mode and operational sex ratio in the evolution of parental care patterns. Colored blocks and bold arrows indicate patterns and transitions of interest in this study.

Evolution of parental care patterns originates through the search of an evolutionary stable strategy maximising the offspring's survival while minimising the cost for each parent [3,4]. Uniparental care and biparental care are two such evolutionary stable strategies [4]. Biparental care evolves in a OSR close to one and is selected if the care provided by both parents is more than twice as efficient as uniparental care [3,35]. Uniparental care often evolves if one parent is scarce in the breeding population (shifted OSR) and, therefore, able to remate easily, while the benefit of the additional caring parent is low [3,35].

Internal parental care with viviparity as the most expensive form is mainly a maternal trait, as the availability of specialised tissues to provide internal care might prevent paternal care. Seahorses and pipefish represent the unique exception with the evolution of male pregnancy [59]. In contrast, in external fertilisation, no physiological constraints are imposed on either sex, which could prevent them from caring. External parental care is thus often provided by either sex.

5. Parental Effects

Conflicts between parents and offspring over how much parental investment per reproductive unit is granted are conditioned by offspring dependence on parental resources and the parents' ability to invest without endangering the own reproductive value [31], while both parties try to maximise their fitness [60]. *Parental effects* provide a tool to alter parental resource allocation depending on the environmental conditions experienced [61] with the aim to boost offspring survival in preparing them optimally for the environmental conditions they are likely to encounter. Parental effects have the potential to change developmental pathways, phenotypic reorganisation and include epigenetic changes leading to differential gene expression in the offspring [61–64].

5.1 Trans-generational immune priming

As an important parental effect, the transfer of immunological experience from parents to offspring (*trans-generational immune priming* (TGIP)) [65] has evolved in both invertebrates and vertebrates. Particularly, in vertebrates, parental provisioning of immunity is crucial for early life stages, since they lack a mature adaptive immune system [66].

In addition to boosting offspring immune responses, benefits of TGIP might include higher growth rates and faster maturation of the offspring [67,68]. However, in non-matching parental-offspring environments, TGIP might be disadvantageous creating high costs [65,66]. Recent studies in invertebrates and vertebrates have shown that TGIP is not limited to mothers, even though sperm was suggested to being too small for transferring more than just the paternal DNA [69–73]. If offspring share the same parasitic environment as the parent in care, selection for paternal or biparental immune priming should exist in species with high paternal reproductive investment, and such effects might even proceed over generations [74].

In fish, the transfer of IgM, lectin and lysozyme via eggs is well documented for many commercially used fish species such as carp, salmon,

plaice, tilapia and spotted wolffish [75–81]. An additional way to transfer immune parameters is enabled by intimate contact of the offspring with immune reactive tissues, such as mucus [72,73,82]. Parental upregulation of mucus production and antimicrobial peptides, as well as micro-nipping from juveniles on parental mucus has been shown in blennies, discus fish and tilapia [73,83,84]. In mouthbrooding cichlids, this intimate contact is also guaranteed over the buccal mucosa and the transfer of immune components is extremely likely [82].

6. Immune system and microbiota

6.1 The teleost immune system

The teleost immune system is in its function similar to the immune system of higher vertebrates [85]. A rapid and efficient innate immune response, elicited within minutes or hours after pathogen contact is followed by a slower but pathogen-specific adaptive immune response. Phylogenetically, teleosts are the oldest vertebrates having the mechanistic basis of the adaptive immune system as observed in higher vertebrates [86]. Compared to the immune system of mammals, teleosts show a more diverse and efficient innate immune system, whereas the adaptive immune system is slower and less versatile [87]. This difference could originate from the temperature dependence, as cellular processes are slower in poikilothermic organisms or be a result of the evolutionary novelty of the adaptive immune system in teleosts [87].

All components needed for both an immediate fast response (innate) and a slow inducible specific response with the development of immunological memory (adaptive) are present in fish [88–91]. Upon pathogenic evasion of the epithelium, pathogen-associated molecular patterns (PAMP) are recognised by pathogen recognition receptors (PRR). Such innate effector cells, i.e. neutrophils, monocytes and macrophages, then activate the complement component pathway and initiate the secretion of various molecules stimulating inflammation and pathogen killing [92]. Furthermore, those cells, together with the complement component start to opsonize and phagocytise the invading pathogen [93]. Presentation of antigen by macrophages as well as cytokine excretion in this phase leads to the activation and differentiation of T cells [93]. Teleosts, generally, possess all prerequisites for an adaptive immune reaction, such as MHC I & MHC II, T- cell receptors and both cytotoxic as well as helper T-cells, but also immunoglobulins and B-cells [88,94]. Although it can take weeks to months to be fully established, teleosts can induce a pathogen-specific immune response, upon secondary exposure this reaction is highly efficient due to the involved immunological memory [93]. Nevertheless, the adaptive immune response in teleost fish is easily influenced by environmental factors (temperature, salinity, oxygen and pH) and changes of those factors might lead to immune suppression [76,95,96].

Due to their aquatic life, teleosts are constantly surrounded by potentially pathogenic microbes. Thus, adequate regulation of an immune reaction is vital to differentiate among commensal microbiota as well as virulent pathogens and fight the latter [93]. The epithelium, covered with commensal bacteria and mucus, represents the first barrier for pathogen invasion, even before innate immune

effectors are activated [97]. Secretion of mucus on surfaces that are in contact with the environment is vital for the fish epithelial immune system (mucosa-associated lymphoid tissue, MALT) due to its antibiotic properties. Mucus includes humoral factors, such as cytokines, lysozymes and lectins but also immunoglobulin's, T- and B-cells that protect the organism by preventing the pathogen penetration.

6.2 Microbiota

Apart from protection against pathogenic microorganisms, MALT is colonised by a vast number of commensal microorganism, the microbiota. The microbiota and the immune system interact reciprocally. Whereas the microbiota keeps the immune system in an active condition, the immune system protects the commensal microbiota from being attacked [98].

Colonisation of the mucosal tissue by a commensal microbiota further prevents pathogenic bacteria to breach the epithelium via competitive exclusion (i.e. secretion of antimicrobial peptides) [99].

Disturbance of the *commensal microbiota* can induce increased autoimmune reactions and a faster inflammation processes. Shifts in the microbial composition may lead to obesity and even malnutrition. Some species of the microbiota are opportunistic pathogens, which occur in a minor proportion in the microbiota [100]. If the homoeostasis is disrupted by stress or antibiotic treatments these microbes can quickly become dangerous disease agents [100].

In fish, the role of microbiota has so far not been studied in depth. However, new germ-free model systems reveal the same interrelation between the microbiota and the immune system as found in higher vertebrates [101]. While information about the gut microbiota is on the rise, information about other tissues and their associated microbiota is still very rare.

The protective abilities of microorganism have been shown in zebrafish and cod by comparing germ-free individuals and those hosting a natural microbiota in their susceptibility towards a pathogenic infection (reviewed in [99]). Differential microbial compositions between males and female wild largemouth bronze gudgeon (*Coreius guichenoti*) might suggest a sex-specific microbiota [102]

The composition of the microbial community depends on the environment, host genetic and immune status, diet and might even fluctuate on a seasonal scale (reviewed in [101]). It is proposed that not only microbial composition but also microbial diversity within the gut of fish is affected by diet, as such microbial diversity increases from carnivores, over omnivores to herbivores [101,103]. Thus an additional function of the microbiota for food digestion, nutrient uptake and fat storage is suggested [104].

The ontogeny of a microbial community starts by colonisation of the egg surface [105]. After hatching, microbes colonise the surface of the larvae and gets eventually ingested and associated in the gut as soon as the larva opens the mouth. Interestingly, studies of larval microbiota composition comparing water and food-associated microbiota with newly hatched larval microbiota detected bacterial species present in the gut but not in the water or the food [99]. Such differential colonisation could hint towards a vertical transfer of microbiota from

the female into the egg (transovarial transfer) similar to vertical microbiota transfer in humans and mice [106].

7. Model system

In my thesis I focused on two teleost families, the cichlids and the syngnathids, both groups display very specific parental care strategies and mating system following a sexual selection gradient. They thus represent enigmatic model systems to study the interrelationship between parental investment, sexual dimorphism and the underlying sexual selection [6].

7.1 Cichlid fishes

Cichlids are found in the great lakes and adjacent rivers of Africa and Central America. The enormous numbers of species endemic to enclosed African lakes have caught the attention of evolutionary biologists as a model system for adaptive radiation, rapid diversification and speciation [107–110]. In Lake Tanganyika, there are estimated around 250 endemic cichlid species of polyphyletic origin, which makes it the most diverse species assembly worldwide [111].

Lake Tanganyika cichlids, as cichlids of other lakes and rivers, are morphological, behaviourally and ecologically highly adapted to their respective habitat [107,112–114]. Several key-innovations are believed to have driven the extreme speciation and niche adaptation in cichlids [115]. The plasticity of pharyngeal jaws is a driver of trophic differentiation [116,117]. Trophic differentiation further allowed cichlids to occupy different niches and to evolve vastly diverse food strategies (see [118] for example). An additional way to explain this immense speciation might be assortative mating [119,120]. Cichlids show various colour polymorphisms, which could lead to reproductive isolation through female choice [120]. It is thus well possible that sexual selection shapes the evolution of this species diversity [109,115,121,122].

Within the cichlids, a vast diversity of reproductive behaviours and parental care strategies has evolved. The ancestral stage was considered to be substrate guarding of the eggs, mostly by the male (see [8]). From this, possibly first as a mere transportation of offspring to different territories as predator avoidance, male mouthbrooding has evolved several times independently from paternal substrate brooding [56,83]. *Biparental and maternal mouthbrooding* are derived stages of paternal care in cichlids [56,83,123].

During mouthbrooding, the eggs and larvae are incubated in the buccal cavity of the parent, until they reach a free-swimming larval stage, after which offspring guarding is often additionally provided [27]. While mouthbrooding is extremely costly for the caregiving part due to low or no food uptake and low respiration [124,125], it provides protection from harsh environment and predation for the offspring [83,107].

In some species females may provide food particles to their offspring during mouthbrooding [126–128]. Nevertheless, the physiological condition of mouthbrooding females is decreased compared to non-brooding females, even if

food particles are ingested during brooding [126]. Furthermore, such behaviour seems rare and the majority of species starve during brooding, potentially, as the cost of brood size reduction for feeding would be smaller than the energy gain by food intake [128].

Adaptations to mouthbrooding are known from both sexes in the form of sex-specific adaptation in cranial form. In the maternal mouthbrooding genus *Tropheus* and *Oreochromis* females have a larger buccal cavity[129,130]. On the other hand, in the biparental mouthbrooder *Eretmodus cyanostictus*, males have a bigger buccal cavity as they guard of the juveniles after hatching, when the offspring are larger and thus need more space in the paternal buccal cavity [131].

Through the high investment in parental care and the existence of a variety of parental care patterns within one family, mouth brooding cichlids are a perfect model to study the adaptation to parental care and the interrelation between investment in parental care and sexual immune dimorphism. To this end, seven species of Lake Tanganyika cichlids with either maternal or biparental mouthbrooding were examined in this thesis.

7.1.1 Maternal brooding species

The maternal mouthbrooding species belong to the tribe of the haplochromini, representing the most species rich-tribe in the Lake Tanganyika species flock [132].

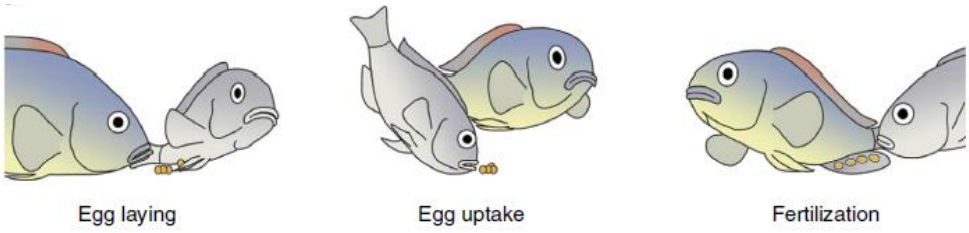

Figure 2: Mating cycle of maternal mouthbrooding hablocromine cichlid fishes.
Figure adapted from Santos et al. 2014

Astatotilapia burtoni is an often used model species for questions in evolutionary biology [133–137]. Inhabiting lake and adjacent rivers, *A. burtoni* exhibits a strong size and colour dimorphism. With an elaborate hierarchical system of social dominance, males are grouped in dominant, subordinate and sneaker males including different colour morphs.

Simochromis babaulti is a rock dwelling lake fish with slight sexual dimorphism feeding on detritus. *S. babaulti* is highly territorial [138].

Tropheus moorii shows distinctive colour morphs within the same species, nevertheless, they do not display sexual dimorphism and are proposed to be monogamous maternal mouthbrooders [129,139]. Tromor females lay extremely large and nutrient rich eggs. Biparental territorial defence and feeding of the offspring was also observed [127,128].

Interochromis loocki the last of the four maternal mouthbrooding species also belongs to the haplochromini and shows similarity in life style to both *S. babaulti* [138].

7.1.2 Biparental brooding species

The three biparental brooding species each belong to the different tribes, Eretmodini (Eretmodus cyanostictus), Ectodini (Xenotilapia spiloptera) and Perissodini (Perrissodus microlepis).

Xenotilapia spiloptera is sand living and feeds mostly of bentoinsects filtered from the sand [138]. Parental care strategies within the *Xenotilapia* are highly interesting, as a transition of maternal mouthbrooding with a polygynous mating system to biparental mouthbrooding with sequential monogamy has occurred supposedly 3-5 times in the last 3 million years [140]. In *X. spiloptera* maternal mouthbrooding is followed by paternal mouthbrooding of larger fry. Post-brooding, the fry is guarded by both parents [27].

Perrissodus microlepis occupies a very distinct trophic niche by feeding on gills of other fish. Morphological adaptation to this lifestyle is a snout tilt to one side. In *Perissodus sp.* biparental fry guarding follows maternal mouthbrooding [141,142].

The third biparental mouthbrooding species was *Eretmodus cyanostictus.* This species has shown to be a monogamous biparental mouthbrooder, where the females incubate the eggs and transfer them to the male after hatching [141,143,144]. Both, males and females vigorously defend their territory on rocky shores, possible to ensure the algal food on the rocks [145]. There is a dimorphism in the size of the buccal mucosa with males having a larger buccal cavity than females [131]. This dimorphism possibly is an adaptation to the more developed and larger brood the male has to carry compared to the female [146].

7.2 Syngnathid fishes

The second clade of teleosts included in my thesis are the ***sex-role reversed syngnathids, displaying male pregnancy*** [147]. In most syngnathids the male is the limiting sex as more eggs are available in the brooding population than can be taken care of by males [148]. Consequentially, sexual selection is inverted; females are limited by the number of mating where males strive for longevity [149]. Such sex-role reversal includes for females to invest in the display of secondary sexual signals which results in immune suppression [48,150].

Male pregnancy has evolved along a gradient with rising complexity. As such there are two main brooding types, the tail brooders “Urophori” and the abdominal brooders “Gastrophori” [147,151]. One striking difference between those two groups is the morphology of the brood pouch. The two genera *Entelurus* and *Nerophis* are the only two abdominal brooding genera, whereas many more genera have evolved among the tail brooders [147]. In the abdominal brooders, the eggs are loosely attached to the abdomen of the male and remain unprotected [151]. The evolution and morphology of the brood pouch in tail brooders are more diverse increasing in complexity from attachment in single compartments over semi sealed brood pouch in *Syngnathus* to the fully closed pouch in sea horses [151,152]. Some species evolved a placenta like structure facilitating transport of nutrients, immune components and allowing osmoregulation and gas exchange [69,74,153].

Seahorses and pipefish are also immunologically extremely intriguing. Lacking a spleen, the organ for the proliferation of T-cells, gut-associated lymphoid tissue (GALT), and the genetics underlying an MHC II based immune system their T-cell mediated adaptive immune system is likely impeded [154,155]. Downregulation of the adaptive immune system in mammalian pregnancy is vital for the survival of the embryo. This immunological rearrangement in the syngnathids could have permitted the evolution of male pregnancy (Roth et al. in preparation).

Both of these features allow disentangling sex from parental care, as the initial investment is independent of the provision of care. Furthermore, the existence of a gradient with rising complexity in paternal pregnancy, allows following the evolutionary cost along this transition in parental pregnancy intensity. With this syngnathids are model organisms for studies of the evolution of parental care, sexual selection and male investment [149,156,157].

Through the range of paternal investment that evolved with the different forms of male pregnancy, the sex-role reversal and the immunological speciality, syngnathids are prime model organisms to study the influence of parental investment on sexual immune dimorphism independent from anisogamy.

Figure 3: Close-up on brood structure in syngnathids. (A) brood pouch of *S. typhle* in late pregnancy, (B) eggs attached to the abdomen of *N. ophidion*. (Picture credit (A) Ola Jennersten and (B) Josefin Sundin. Adapted from Braga-Goncalves et al. 2016)

To this end, I used three species of syngnathids along a gradient of increased parental investment.

Nerophis ophidion is an abdominal brooder with the eggs attached to the male abdominal [151]. Compared to the other two species paternal investment in the offspring is relatively low. *N. ophidion* is polyandrous, thus the brood of each male consist only of the eggs of one female. The female, limited in her reproduction by the caring capacity of the males, shows strong blue coloration in the brooding season and is larger than the male [150,156].

Syngnathus rostellatus is a tail brooder with a semi-sealed brood pouch and placenta-like structure [151]. In this comparison, they represent the intermediate parental investment mode. *S. rostellatus* is polygynandrous, as the

male accepts eggs from several females during one brooding but still limits the reproductive success of the female [158]. Sexual dimorphism is restricted to the female being slightly bigger than the male.

Syngnathus typhle, although similar to *S. rostellatus* in mating system and brood pouch type, this species is the one with the relatively highest paternal investment through bigger eggs and longer pregnancy. In *S. typhle* biparental transfer of immune components has been shown to exist in and even consists over generations [69,74,159]. Additionally, body size but also MHC I and cues about the mate's health, guide mate choice in *S. typhle* [157,160,161].

THESIS OUTLINE

This thesis is structured in three chapters each in form of a manuscript containing an abstract, introduction, material & methods, results and a discussion. The first chapter is under review in "*Evolution*", the second chapter will be submitted after data of preceding studies are published and the third chapter is under review in "*BMC Evolutionary Biology*".

I investigated how the interrelationship between parental investment and sexual immune dimorphism shape the evolution of parental care strategies within the cichlids and syngnathids. To understand why parental investment is displayed in such diversity in the animal kingdom, I assessed evolutionary and provisioning costs of parental investment in male pregnancy, biparental and maternal mouthbrooding. Additionally, to address the importance of parental investment, I tested for maternal effects transferred via mouthbrooding, focusing on the transfer of immunological information. I could identify differential allocation of resources between the two parental care strategies, mouthbrooding and male pregnancy, in both provisioning and evolutionary costs of parental care. Further, my data suggest that both parental care and mating system influence sexual dimorphism. I was not able to detect transfer of immunological information via mouthbrooding; rather, my data imply that stressed mothers are impeded in their resources that can be allocated to boost their offspring survival.

Chapter I

I compared the interrelationship of parental care systems, sexual immune dimorphism and buccal microbiota in biparental and maternal mouthbrooding Lake Tanganyika cichlids. To this end, I measured candidate gene expression, cellular immune parameter and analysed buccal microbiota of seven cichlid species with either maternal (four species) or biparental (three species) mouthbrooding. According to sexual selection theories, males are limited by the number of matings, whereas females strive for longevity. This leads to sex-specific allocation of resources towards either secondary sexual signals in males or towards self-maintenance and elongated lifespans in females. These differences generate sexual dimorphism. The larger the difference in resources allocation between the sexes the more accentuated is the sexual dimorphism. In maternal brooding species, sexual selection on males is strong through female choice and intra-sexual competition, while costs of parental care are high on the female. In species with biparental mouthbrooding sexual selection on males is attenuated, as the investment in secondary signals is decreased and costs of parental care are shared between the sexes. Maternal brooding species were thus expected to show a stronger sexual immune dimorphism and sex-specific buccal microbiota than biparental species. This expectation was confirmed in my

thesis; cichlids have brooding-mode specific gene expression and microbiota communities. In addition, my results suggest a sexual dimorphism in cellular immune parameter and candidate gene expression in maternal but not biparental brooding species. Interestingly the identified sexual dimorphism relates to both mating system and parental care patterns.

Chapter II

In the second chapter of my thesis, I focused on the interrelationship of paternal care intensity and sexual dimorphism in three sex-role reversed syngnathus species with different male pregnancy intensities. Additionally, I investigated the resource-allocation trade off between parental pregnancy and the immune system regarding evolutionary costs of intense paternal investment and transient provisioning costs during pregnancy. To this end, I measured immune cell parameter and candidate gene expression along a gradient of paternal care intensity from *Nerophis ophidion* (external pregnancy), over *Syngnathus rostellatus* (short internal pregnancy; small eggs) to *Syngnathus typhle* (long internal pregnancy; large eggs). I measured females and non-pregnant males to see differences in sexual immune dimorphism, whereas differences in sex-specific allocation of resources are supposed to increase along this gradient. Within species comparison of the measured parameters during pregnancy (non-pregnant, pregnant, after parturition) yield insight into the provisioning costs of each of the paternal pregnancies. By comparing the different male reproductive stages between the species, I could assess the evolutionary cost of such intense and specialised care. The sexual dimorphism was strongest in the species with most intense paternal pregnancy. Furthermore, intense paternal care both influenced the provisioning costs as well as the evolutionary costs of parental investment. As such, trade-offs between self-maintenance and reproduction are strongest in the species with most intense care both compared to non-pregnant males of the own species (provisional costs) as well as compared to the same reproductive stage in the other species (evolutionary costs).

Chapter III:

In chapter III, I measured provisioning costs of maternal mouthbrooding and addressed the potential transfer of immunological information to the offspring via the buccal mucosa. To this end, females of the mouthbrooding African cichlid *Astatotilapia burtoni* were immunologically challenged with heat-killed Vibrio or left naïve. Both challenged and naïve females reproduced and were sampled at two time points, shortly after egg uptake or when mouthbrooding was completed. This allowed disentangling resource allocation towards the immune system during reproduction versus parental care (brooding) under ambient (naïve) and immunologically stressed (immune challenge) conditions. After mouthbrooding, juveniles were exposed to a bacteria challenge either homologous or heterologous to the maternal exposure to assess a potential

transfer of immune components and its specificity via the buccal mucosa. My results suggest distinct provisioning cost of reproduction and mouthbrooding that are, however, both traded off with the immune system. In mouthbrooding females, an immunological challenge induced higher stress response possibly through the two-fold cost of resource allocation towards brooding and immune defence. Juveniles from challenged mouthbrooding females showed a lower expression of candidate genes than juveniles from naïve females. Thus, maternal stress rather seems to impede juvenile immune response instead of boosting offspring immune responses.

CHAPTER I

The mating system determines resource allocation into buccal microbiota and immune defence in mouthbrooding cichlid fishes

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Abstract:

Sexual dimorphism is founded upon a resource allocation trade-off between investments into reproduction *versus* other life-history traits including the immune system. In conventional parental care roles, males maximise their lifetime reproductive success by allocating resources towards sexual selection, while females achieve this through prolonging their lifespan. Here, we examine the interrelation between sexual dimorphism and parental care strategies in closely related maternal and biparental mouthbrooding cichlids from East African Lake Tanganyika. More specifically, we measured cellular immune parameters, examined the relative expression of 28 immune system and life history related candidate genes and analysed the microbiota composition in the buccal cavity. According to our predictions, maternal mouthbrooders are more sexually dimorphic in immune parameters and mucosal microbiota than biparental mouthbrooders, which has possibly arisen through a differential resource allocation into parental care *versus* secondary sexual traits. Biparental mouthbrooders, on the other hand, which share the costs of parental care, feature an upregulated adaptive immune response and stronger antiviral properties, while their inflammation response is reduced, suggesting a differential resource allocation trade-off between the two modes of mouthbrooding. Overall, our results suggest that sexual dimorphism relates to mating systems (monogamy *versus* polygamy) rather than parental care systems (maternal *versus* biparental mouthbrooding).

Keywords: sexual dimorphism, parental investment, mouthbrooding, cichlids, buccal microbiota, sexual immune dimorphism

Abbreviations: LRS = lifetime reproductive success, MB = mouthbrooding, BPMB = biparental mouthbrooding, MMB = maternal mouthbrooding

Chapter II

Parental investment and immune dynamics in sex-role reversed pipefishes

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Abstract

Parental investment and sexual dimorphism are closely linked over sexual selection. That females provide costly eggs and males small sperm forms the basis for sexual selection, in which the female usually limits reproductive success. Leading to a sexual selection gradient, with male competition and female choice, which might be enhanced by post-zygotic investment. In sex-role reversed species an inverted gradient of sexual selection results in female competition and male choice, especially when including intense paternal care. Sex-role reversed species provide excellent grounds for the investigation of the interrelation of parental investment and sexual dimorphism in a sex-independent way.

We investigated the evolutionary and provisioning costs of parental investment the resulting interrelationship with sexual dimorphism in three species of sex-role reversed pipefishes (syngnathids) along a gradient of paternal investment intensity. We propose sexual dimorphism to increase with paternal investment due to intensified sexual selection. Additionally, we suggest distinct resources are allocated towards the provisioning and the evolution of paternal care revealed in trade-offs with other life-history traits, positively correlated with the parental care intensity.

To this end, we measured cellular immune parameters and candidate gene expression (immune, metabolism and pregnancy related genes) in *Nerophis ophidion* (low paternal investment), *Syngnathus rostellatus* (intermediate paternal investment) and *Syngnathus typhle* (high paternal investment). Comparison of females and non-pregnant males reveals the extent of sexual dimorphism. By within and between species comparison of males in different reproductive stages both evolutionary and provisioning costs were assessed

As predicted, sexual dimorphism is strongest in the species with highest paternal investment. During paternal pregnancy, we detected species-specific resource allocation towards reproduction in all species but strongest in the species with the highest parental investment. Evidence for an evolutionary cost of paternal investment, independent from on-going parental care, was identified, as effects between differential reproductive stages were strongest in the species with the highest parental investment. Our data thus suggests a parental investment intensity specific allocation of resources and a connection between parental investment and sexual dimorphism.

Keywords: Sex role reversal, sexual dimorphism, cost of parental care, sexual selection gradient, syngnathids

Chapter III

Parental Investment Matters for Maternal and Offspring Immune Defense in the Mouthbrooding Cichlid *Astatotilapia burtoni*

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Abstract:

The evolution of parental investment was facilitated over fitness benefits due to increased offspring survival. However, if concurrently to a reproductive event, additional stressors ask for an allocation of resources, negative consequences on both the parental and the offspring condition can be predicted, as parental investment is a costly trait. While the immune system is reflecting parental stress conditions, parental immunological investments also boost offspring survival via the transfer of immunological substances (trans-generational immune priming). If demands for individual defence against parasites and demands for immunological investment into offspring are simultaneously high on the maternal side, females may suffer reduced immunological activity.

We investigated this prediction in a common garden experiment using the East African cichlid *Astotatilapia burtoni* featuring an extreme parental investment strategy: female mouthbrooding. Prior to mating, females were exposed to an immunological activation, while others remained immunologically naive. Females were then permitted to mate and take up the fertilised eggs into their buccal cavity. Mouthbrooding was either completed, or eggs were raised artificially. Correspondingly, immunological status of females was either examined directly after reproduction or after brooding had ceased. Offspring from both groups were exposed to immunological challenges to assess the extent of trans-generational immune priming. As proxy for immune status, cellular immunological activity and gene expression were determined.

Both reproducing and mouthbrooding females allocate their resources towards reproduction. While upon reproduction the innate immune system was impeded, mouthbrooding females rather showed an attenuation of inflammatory components and elevated stress levels. Juveniles from challenged mouthbrooding females had a reduced expression of candidate genes, implying a limitation of trans-generational plasticity when parents experience stress during the costly reproductive phase.

Our results provide evidence that parental investment via mouthbrooding is beneficial for the offspring. However, both parental investment and the rise of the immunological activity upon an immune challenge are costly traits. If applied simultaneously, not only mothers seem to be impacted in their performance, but also offspring are impeded in their ability to react upon a potentially virulent pathogen exposure.

Keywords: parental care, sexual dimorphism, trans-generational immune priming, immune system, teleosts, phenotypic plasticity, gene expression

SYNTHESIS

In my thesis, I investigated the effect of *parental investment on sexual immune dimorphism (Chapter I & II).* To detect the interrelationship between sexual immune dimorphism and intense parental care, I measured cellular immune parameters and candidate gene expression in three species of sex role reversed syngnathids with paternal pregnancy. The same measurements in combination with an analysis of the buccal microbiota were applied in seven species of African cichlids with either maternal mouthbrooding (MMB; four species) or biparental mouthbrooding (BPMB; three species).

I assessed the *evolutionary costs of parental care on the parental immune system (Chapter I & II).* Comparison of the measured parameters (cellular immune parameter, candidate gene expression & buccal microbiota (only cichlids) between non-brooding individuals from different parental care intensities (syngnathids) and different MB modes (cichlids) yields information about the costs associated with the evolution of distinct parental care strategies to enlighten the evolutionary transition of parental care patterns and parental investment.

Additionally, I evaluated the *provisioning costs of parental care on the immune system (Chapter II & III).* Within species comparison of the immune competence in different reproductive stages (no reproduction with reproduction & MB or non-pregnant with pregnant and after parturition males) can indicate provisioning costs of parental care. This allows drawing conclusions about a possible resource allocation between parental care and the immune system. In the maternal mouth brooding cichlid *Astatotilapia burtoni*, I additionally measured provisioning costs of parental care (reproduction & MB) under immune challenge to assess the double cost opposed on female *Astatotilapia burtoni* by establishing an immune reaction and providing parental care.

Further, I examined the *possibility and specificity of trans generational immune priming through mouthbrooding (Chapter III).* To test for the existence and specificity of TGIP through the buccal mucosa during MB, juveniles of challenged or naïve *Astatotilapia burtoni* were either mouthbred or reared artificially. After reaching a free-swimming stage, those juveniles were then subjected to an immune challenge with bacteria homologous or heterologous to the maternal challenge. Candidate gene expression of those juveniles gave insight into the transfer and specificity of immune parameter.

1. Does sexual dimorphism in immune functions and microbiota depend on parental care patterns?

In my thesis, I investigated sex-specific resource allocation towards immune defence in *three syngnathid species* with differential investment in paternal care. I hypothesized that sex-specific investment into the immune system depends on parental care intensity, tested by comparing the immune competence of females and non-pregnant males in species with differential investment intensities. I found a sexual immune dimorphism in a higher adaptive

to innate immune cell ratio in males and elevated immune gene expression in males compared to females. Resource allocation towards the establishment of secondary sexual signals and away from the immune system could be a reason for this decreased immune competence in females. This is consistent with previous studies on the sexual immune difference in *S. typhle* and *H. erectus* finding an immune-suppressed female [48,248].

In a species wise comparison, the expectation was that sexual immune dimorphism decreases with decreasing parental care intensity. *N. ophidion*, in this case, was expected to have the weakest sexual immune dimorphism followed by *S. rostellatus* and *S. typhle*. Candidate gene expression interestingly yielded another result, as such no sexual immune dimorphism was found in *S. rostellatus* and *N. ophidion* where as the gene expression pattern in *S. typhle* show sex-specific allocation of resources. Parental investment can explain most of the existing sexual immune dimorphism. However, also differences in mating system could account for these findings. *N. ophidion* males receive all eggs from one female, while in both syngnathus species males accept eggs from several females [150]. This could potentially lower the sexual selection in syngnathus species and could therefore lead to a reduced sexual immune dimorphism. The combined effect of weaker selection and shorter pregnancy time (compared to *S. typhle*) in *S. rostellatus* could add up explaining the non-observes sexual immune dimorphism in this species.

In *cichlids comparison of males and females with differential MB modes* was expected to show a distinct sexual immune dimorphism in maternal brooding species, as selection on males should lead to allocation of resources towards secondary sexual signals, whereas females rather strive for longevity [30,36,44]. In biparental brooding species, selection is supposedly attenuated in both sexes, due to shared parental care and weaker sexual selection, leading to a less distinct sex-specific allocation of resources.

Cellular immune measurements suggest sexual immune dimorphism to be present in MMB but not in BPMB. Females of MMB species show a higher adaptive to innate immune cell ratio and a higher ratio of active adaptive immune cells than males. Hierarchical clustering of candidate gene expression, however, suggests a more complex pattern behind sex-specific resource allocation. The sexes of all BPMB species cluster together, which implies that there is no difference in expression patterns between BPMB males and females. In MMB species, the expected sexual dimorphism was to be visible in a distinct clustering of males and female of each species, indicating a sex-specific gene expression pattern. However, only half of the species have sex-specific gene expression (*S. babaulti* & *I. lookii*). These sex-specific differences in candidate gene expression could eventually be attributed to sex-specific selection through a MMB mode. In the other two species (*T. moorii* & *A. burtoni*) the sexes cluster together underlining that no dimorphism in candidate gene expression can be assumed. One of the MMB species, *Astatotilapia burtoni*, has in the laboratory been shown to be immunologically dimorphic (Chapter II) [134], hence, the sex-independent gene expression pattern is striking. One possible explanation might

be found in the highly complex social system observed in *A. burtoni*. Dominant males and subordinate males mix within a population, whereas only dominant males are territorial and have a higher reproductive success, with influences in hormonal regulation and gene expression [302–304]. Hence, a potential sexual immune dimorphism between females and the reproductive active dominant males could have been masked by gene expression in subordinate males. In *Tropheus moorii*, the mating system might explain differential sex-specific resource allocation patterns. Recently proposed to be a serially monogamous MMB with post mouthbrooding paternal care [139], the reproductive success of both sexes is limited by mouthbrooding resulting in weaker sexual selection and with this diminished sexual immune dimorphism.

The microbiota of the buccal cavity did not differ in diversity or composition between the sexes in either MB mode. This is astonishing, as sex-specific differences in gut microbiota composition have been found in humans and mice suggesting a hormonal influence on the gut microbiota [187,305,306]. However, selection on the microbiota of the buccal cavity and the gut possibly differs, as the environmental influence is stronger on the first. It is tempting to speculate that the gut microbiota might be more specialised, at least during times of no brood care. Analyses of possible shifts in microbiota composition of the buccal mucosa at differential brooding stages would thus be very interesting.

Both, *syngnathids and cichlids,* show that patterns of sexual immune dimorphism dependent, at least partially, on high parental investment. It can be concluded that higher parental investment and the resulting shift in sexual selection is influencing the degree of sexual immune dimorphism observed in a species. This data is thus consistent and even extends the theory of selection-specific allocation of resources towards immunity [44,252].

Nevertheless, it seems that additional factors have to be taken into account when analysing the interrelationship between sexual immune dimorphism and parental investment intensity. One of these might be hormonal regulation of reproduction and social hierarchy, which might influence the immune system. Additionally, hormonal changes during the brooding cycle could influence sex-specific immunocompetence.

Further, mechanisms determining sexual selection might influence sexual immune dimorphism next to parental investment. In the cichlid *T. moorii* (MMB and monogamous) and in the syngnathid *N. ophidion* (low investment and polyandrous), mating system seems to interfere with the pattern expected by sex-specific parental investment alone. In monogamous species (*T. moorii*), sexual selection is weak resulting in lower sexual dimorphism and thus possibly also in less distinct immune dimorphism [35], the contrary of what was expected in MMB species. In polyandry on the other hand, sexual selection on the female is increased leading to a more distinct immune dimorphism, and thus possibly explaining the pattern observed in *N. ophidion* in spite of the low paternal investment.

2. The cost of parental investment:

2.1 Evolutionary costs of parental investment:

In *pipefish* I assessed evolutionary costs of adaptation to male pregnancy by comparing the *same reproductive stages in three different species* with increasing investment in parental care. According to my hypothesis, trade offs between investment in parental care and the immune system should be strongest in the species with the highest care, i.e. in *S. typhle* and decrease over *S. rostellatus* to *N. ophidion*. Indeed immune cell measurements suggest a higher investment in cellular immunity in *S. typhle* and *S. rostellatus* compared to *N. ophidion*. Candidate gene expression reveals a shift during pregnancy; non-pregnant *S. typhle* and *N. ophidion* males show a similar gene expression pattern that is distinct from non-pregnant *S. rostellatus* males. This changes in pregnant males and males after parturition, here *S. typhle* have an expression pattern distinct from the other two species. This shift during pregnancy and after parturition suggests differential evolutionary costs associated with pregnancy in *S. typhle*. Deduced from the complexity of the brood pouch and energy allocated into the offspring [70,151,237], I provide evidence for higher evolutionary costs in *S. typhle* compared to the other two species. Specific adaptations to this unique male pregnancy in syngathids range from morphological to genetic changes adaptations within the male [151,307,308]. Such adaptations could eventually be costly, due to resource allocation trade off or antagonistic expression of genes and thus impede life history. Through the longer more specialised pregnancy in *S. typhle* such resource allocation trade offs might be stronger thus explaining the shift in expression pattern during pregnancy in *S. typhle* as a potential shift in life history. The fact, that *S. rostellatus* does not exhibit this change might be accounted to the plasticity of brood pouch function in the syngnathids, the shorter pregnancy phase and smaller eggs [151,153].

In *cichlids*, BPMB species display an increased cellular innate immune response compared to MMB species. The candidate gene expression data hint towards a better T-helper cell response in BPMB species. As MMB species do not show a more active adaptive immune gene expression, this hints towards a resource-allocation trade off between investment in parental care and investment in the immune system. The lower expression of the pro-inflammatory integrin in BPMB might be an adaptation to egg/larval swapping during biparental care. High inflammation responses after brood swapping might be harmful for both the larvae and the parent.

In the β-diversity analysis of the buccal mucosa microbiota, Indicator OTUs of MMB species were a mixture of gram-positive and gram-negative bacteria, whereas indicator OTUs of BPMB species all belonged to the gram-positive bacteria. Both bacteria types may evolved different solutions for interacting with the host immune system and possess the abilities to stimulate differential immunological pathways. Striking in this study is that gram-positive bacteria are rather associated with the stimulation of monocytes to produce interleukin, whereas gram-negative bacteria have a rather anti-inflammatory

property [218]. Combined with the gene expression, this could suggest a possible immunomodulatory function of the buccal mucosa microbiota, especially through the close proximity of the measured organs (gills & buccal cavity).

The prevalent genera *Acinetobacter* and *Aeromonas* in the buccal cavity of the MMB species could indicate an induced stress level, as both genera are associated with stress in the epithelial mucus of salmonids [210].

Concluding, my data indicate that MMB species are generally of lower immunological condition and might be more affected by stress than BPMB species. This is likely due to the high investment in parental care by the female and the high investment in secondary sexual signals by the male.

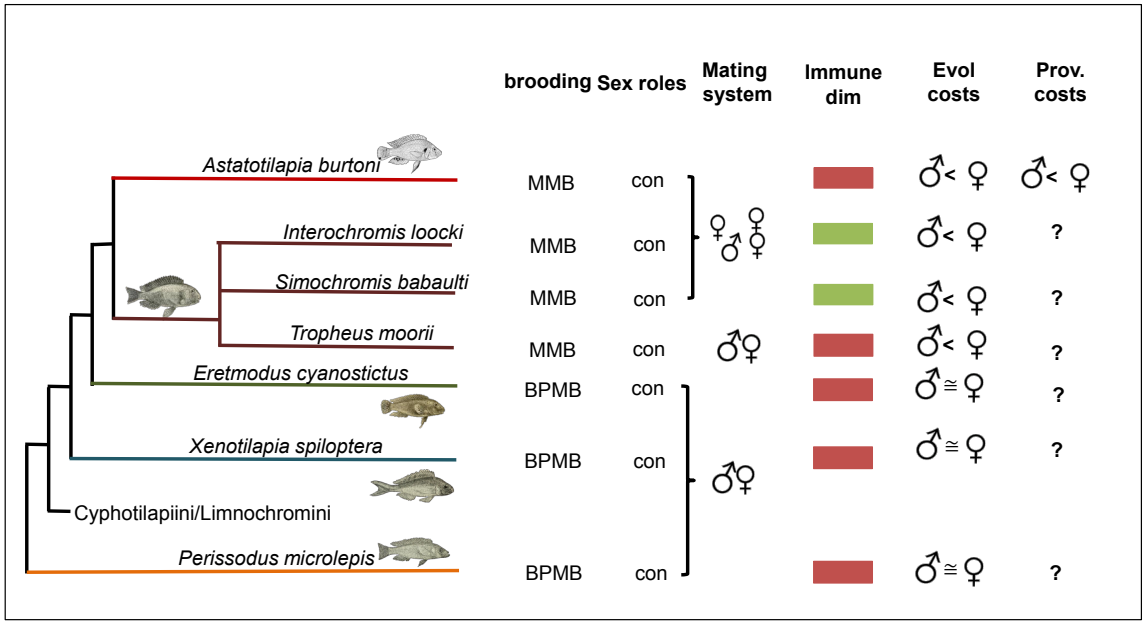

Figure 1: Schematic overview of the Results in relation to brooding mode, sex-roles and mating system. Sexual immune dimorphism is absent in species with a red block and present in species with a green block. In both, evolutionary and provisioning costs, the pictograms show direction of higher cost. Conventional sex roles = com. (Phylogeny is derived from Meyer et al. 2015)

In a direct *comparison of those two systems,* I could reveal that the evolutionary costs of high parental investment can be detected in the immune system. Both syngnathids and cichlids show signs of immunological adaptation to their respective parental care mode.

One important immunological adaptation for parental care in an immunologically active tissue, such as the buccal cavity or the brood pouch, is the prevention of allograft rejection [309,310]. In syngnathids it was proposed that the loss of a functional MHC class II pathway might be such an adaptation to internal brooding [155]; Roth et al., in preparation). The here observed shift in candidate gene expression in *S. typhle* over pregnancy could thus have a similar reason. In cichlids, the evolutionary costs during brooding where not assessed as only non-brooding individuals were sampled. Hence, the upregulation of the innate immune system and T-helper cell response in BPMB species cannot be

attributed as adaptation to brooding. Nevertheless, the same upregulations are, in mammals associated to pregnancy (reviewed in [310]). By very bold speculation one could interrelate these results possibly hinting towards a general immunological adaptations to internal parental care, even in mouth brooding cichlids. Of course this would first have to be tested by comparing brooding and non brooding individuals of both BPMB and MMB species, which could be part of a further experiment.

2.2 Provisioning costs of parental care:

Provisioning costs of immediate investment in parental care during brooding, reproduction and pregnancy can be seen as an allocation of resources towards traits needed for parental care and away from other costly life-history traits such as the immune system [180].

In *syngnathids,* males decrease the ratio of adaptive to innate immune cells towards the end of the parental care phase. In comparison males after parturition downregulate the expression of adaptive, innate and complement component genes compared to non-pregnant males. This indicates a general decrease in immune competence during the course of pregnancy leading towards a lower immune capacity after parturition. This effect seems to be driven by males with the highest paternal investment (*S. typhle*) and, astonishingly, males with the lowest paternal investment (*N. ophidion*). In *S. rostellatus*, the species with intermediate paternal investment, no provisional costs have been found. Thus my results suggest a species-specific pattern of provisional cost during paternal pregnancy.

The rather striking result of no provisioning costs in *S. rostellatus* could eventually be partially accounted to the female. The amount of nutrients provided with the egg might lead to a decreased need of paternal provisioning via the placenta. However, that eggs in *S. rostellatus* are smaller than the ones in *S. typhle* may rather speak against this argument. Therefore, the lacking evidence of provisioning costs on the paternal side in *S. rostellatus* could at least partially be explained through uptake of by maternally provided nursing eggs (eggs providing nutrients for the brood). By radioactive labelling of eggs it has been shown, that those nutrients are absorbed by the male and integrated in the brood pouch, liver and muscle tissue [311]. While this was as yet only investigated in *S. typhle*, the benefit of nutrients for the father from unfertilized eggs might be higher in *S. rostellatus*, which should be evaluated in a future experiment. Hence, differences in egg quality and provisioning of additional nutrients from the females could lead to differences between *S. typhle* and *S. rostellatus* [153,312].

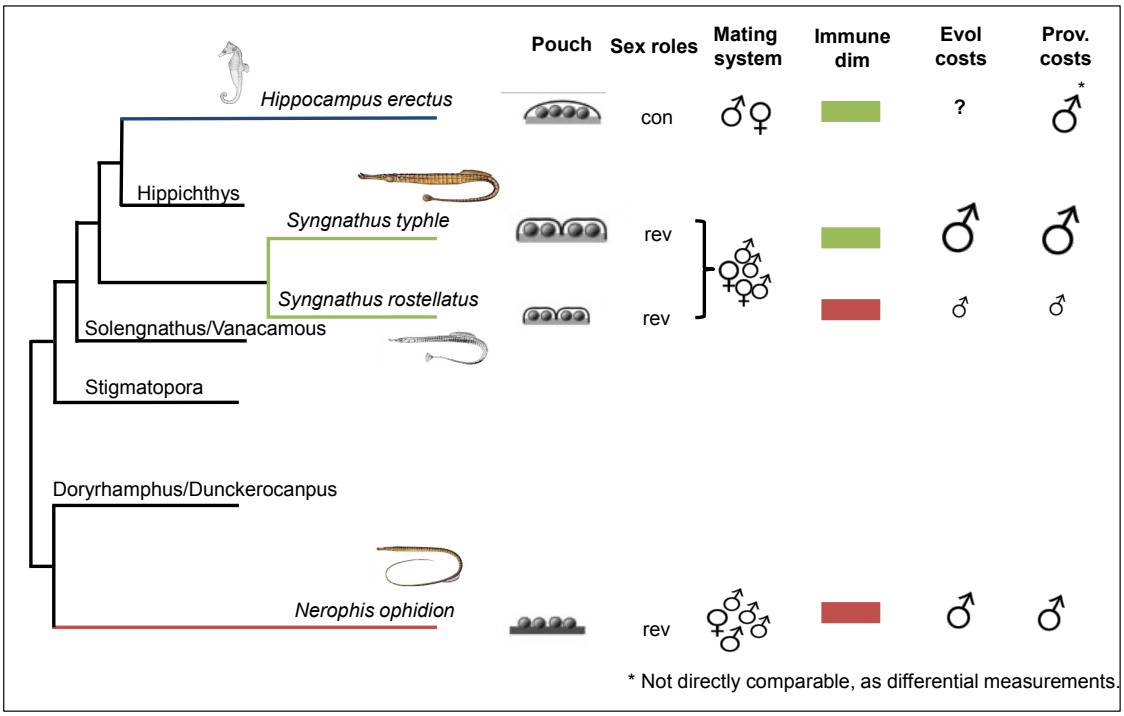

Figure 1: Schematic overview of the Results in relation to pouch morphology, sex-roles and mating system. Sexual immune dimorphism is absent in species with a red block and present in species with a green block. In both, evolutionary and provisioning costs, the size of the pictogram is relative to the strength of the respective cost. Conventional sex-role species = con, sex-role reversed species = rev. (Pouch pictograms & phylogeny is adapted from Stölting et al. 2007. Results from H. erectus are integrated from Lin et al. 2016)

Further, *S. typhle* and *S. rostellatus* males, in contrast to *N. ophidion*, might have the possibility to selectively choose the number of eggs to be taken up by accepting or rejecting broods from females, as often broods contain eggs of several females [25,313]. If provisioning costs increase with number of offspring, a reduction of egg numbers incubated during one pregnancy interval might be favourable, as shown in *S. typhle* [314]. Assuming a similar ability of brood reduction in *S. rostellatus* together with the usually smaller eggs would be an explanation for the non-existence of both provisional and evolutionary costs in *S. rostellatus* found in this thesis. Thus if the brood pouches of the sampled *S. rostellatus* where not entirely filled less resources would have to be allocated towards pregnancy, lowering the provisional costs. As neither the numbers nor the percentage of brood pouch fullness was quantified during the experiment, this remains speculative but should be taken in to account in further studies.

Additionally, it has to be taken in consideration, that the transfer of nutrients and energy to the offspring, could explain the higher amounts of antimicrobial proteins and the higher metabolism in males after parturition. As such, the upregulation of the C-type lectin found during pregnancy is consistent with current results in *Hippocampus* [246] and in *S. typhle* [247] , where an protective function for the embryos in the brood pouch was proposed for this lectin. Higher metabolism in pregnant males, could result through the need of more blood flow towards the highly vasculinised [315] brood pouch in pregnant males. Where as lower cellular adaptive immune Reponses during pregnancy could eventually

result from a resource allocation trade off between the male immune system and the provision of the offspring in the brood pouch.

A recent study provides additional insight into the resource allocation trade off between the immune system and male pregnancy in *Hippocampus erectus* [248]. Opposed to the results from this thesis, during pregnancy *H. erectus* males upregulated their immunological activity. Nevertheless, in comparison with the three species used in this thesis, *H. erectus* is not sex-role reversed [234] but has conventional sex roles. Such distinct mating systems change evolutionary stable strategies due to sexual selection and may have profound implications on the suggested resource-allocation trade off both before and during pregnancy. During courtship, *H. erectus* males have investment in both display of secondary sexual signals and preparation of the brood pouch. Both investments are in a resource allocation trade-off with investment in the immune system resulting in a downregulated immune system during courtship. After mating, reduction of investment in secondary sexual signals reduces this trade-off, which could coincide with the induced immune competence in *H. erectus* after mating during early pregnancy. Provisioning costs of paternal can also be seen in *H. erectus* as immune parameters decrease with proceeding pregnancy [248].

In *Astatotilapia burtoni, reproduction* (egg production and mating) leads to a reduced proportion of adaptive to innate immune cells and a downregulation of genes from the innate immune system, metabolism genes and a sex hormone. It is possible that the lower adaptive to innate immune cell ratio, as compared to non reproducing individuals, might be due to a production of innate immune cells that are transported towards the buccal mucosa. The upregulation of a stress-induced heat shock protein in females during reproduction hints towards an elevated stress level though egg production and preparation of the buccal mucosa. Interestingly, immune challenges with heat-killed *Vibrio* do not affect the immune system of reproducing females differently than non-reproducing females. Hence, a double cost of reproduction and investment in the immune system during challenge was not found.

In contrast to this, in *mouthbrooding Astatotilapia burtoni* effects of both mouth brooding and immune challenge as well as an interaction of both factors have been identified. As such, mouthbrooding lowers the adaptive immune cell activity and provokes a higher ratio of adaptive to innate immune cells. Further, mouthbrooding individuals downregulate the expression of the gene 'cortisol receptor', a glucocorticoid receptor responsible for downregulation of cortisol level, thus possibly resulting in higher cortisol levels in mouthbrooding females. High cortisol levels reflect acute stress and have a suppressive impact on the immune system. This is in line with the downregulation of the thrombin receptor during mouthbrooding indicating a reduced inflammation response. Thus provisional costs of mouth brooding in *A. burtoni* are related to a resource-allocation trade off between investment in mouthbrooding and investment in the immune system and general physiological conditions. An additional explanation

for the downregulated inflammation response might be an adaptation of mouthbrooding in order to prevent rejection of the juvenile in the buccal mucosa.

The *double cost of mounting an immune response and investment in mouthbrooding* leads to a downregulation of lectine and chemokine receptor, both important in immune system signalling. Additionally, lectine and chemokine receptors might be important for the antibacterial and antifungal protection of the offspring within the buccal mucosa.

Mouthbrooding alone impedes the female inflammation response and elevates stress levels, whilst a double cost seems to impede immune system signalling and possibly also sufficient buccal mucosa preparation for the offspring.

Costs of providing mouthbrooding have been intensively studied concerning oxygen metabolism [17] and starvation [125] as both oxygen and food uptake is impeded by brooding eggs within the buccal cavity. As such, costs of mouthbrooding have been measured as elevated respiration, surface breathing, decrease in condition factor and body fat [124]. A decrease in general physiological condition and the involved drop in resource availability during mouthbrooding as well as the higher stress response identified could be costs due to starvation and lower oxygen metabolism.

Both, *male pregnancy and maternal mouthbrooding* involve a shift in resource allocation towards parental care and away from investment in the immune system. However, this trade off between investment in parental care and investment in the other life-history traits can be assigned to different pathways when comparing male pregnancy versus mouthbrooding. While mouthbrooding females suffer from a higher acute stress and a lower inflammation response, pregnant males and males after parturition have a higher metabolism and higher antimicrobial protein secretion but lower cellular adaptive immune response. This suggests that while in both parental care strategies parents suffer an impeded immune function, the identified costs are specific to the parental care strategy and the species examined.

3. Transfer of immune components in Astatotilapia burtoni:

Transfer of immune components integrated in the egg, has been found in many species. Additionally to this direct transovarial transfer, post-zygotic close contact between the offspring and the parent has been shown to boost the offspring immune system [73,82]. In Chapter III, I investigated the existence and specificity of transfer of immune components via close contact with the maternal buccal mucosa. To differentiate between effects arising from integration in the eggs and effects arising from transfer via the buccal mucosa, I intended to compare artificially reared offspring and mouthbred offspring. Test for specificity was done by homologous (*Vibrio*) or heterologous (*Tenacibater*) immune challenge of the offspring from previously challenged females (*Vibrio*). The high mortality rate of juveniles from the artificially raised offspring supports the assertion that mouthbrooding is adaptive.

The effects of the maternal condition on mouthbred juvenile performance were stronger than the effects of juvenile challenge underlining the importance of maternal effects. I expected an upregulation of immune genes in the juveniles of homologously challenged females due to TGIP. In contrast, most juveniles show a downregulation of immune system genes and developmental genes upon homologous bacteria exposure (same bacteria as their mothers). These results contradicts most previous studies about transfer of immune parameter to date [66,240,257,260,316]. Mouthbrooding females that are exposed simultaneously to an immune challenge are stressed through a resource allocation trade off between investment in mounting an immune response and investment in the current reproductive event (see above). Maternal stress could be one factor leading to impeded immune functions in the offspring [301]. In this case, environmental stress, through the maternal immunological activation, could lead to lower quality offspring.

An additional reason for the negative impact of maternal stress on mouthbred juveniles could be the buccal mucosa environment. During stress, the mucosa is proposed to be disrupted leading to disbiosis and potentially illness [100]. Stress, induced by the double cost of investment into reproduction and immune system, might thus be disrupting the female buccal microbiota, leading to colonisation and growth of pathogenic and opportunistic microorganisms [100]. Juveniles being raised in the buccal cavity will thus face a potentially hazardous microbiota that could negatively impact offspring development, induce stress and delay immunological maturation [99,184]. I suggest examining this in the future by comparing microbial buccal communities in different reproductive stages (i.e. over brooding), as well as gene expression and immune defences in both adults and their offspring.

4. Conclusion

In this thesis, I detected a profound interrelation between sex specific investment in parental care and sexual immune dimorphism. However, this presumably simple relation is complicated by impact of the species-specific mating systems and social behaviour that both seem to influence sexual immune dimorphism.

I could demonstrate that both the evolutionary costs and the provisioning costs of parental care influence the immune system of the care-giving parent. Those costs differ among species and parental investment mode. Additionally, I found a reproductive stage-dependent resource-allocation trade off between investment in the current reproductive event and the self-maintenance during an immune challenge.

I did not detect trans-generational immune priming via the buccal mucosa, rather it seems that maternal stress lowers offspring performance, indicating the possibility for maternal effects via mouthbrooding.

THE BIG PICTURE

So far, the interrelation between sexual immune dimorphism and parental care patterns has not been addressed thoroughly. This is surprising given the clear connection between immune competence and sexual selection and thus the dependency on parental investment. The great difficulty faced in this study, but also in past and future work, is the disentangling of mating system and parental care patterns, as both are related to the same concepts and are closely intermingled.

While the evolution of transitions in parental care are being studied to determine the origin of specific parental care patterns, the evolutionary costs of parental care need further investigation. Studying adaptations to parental care patterns, the underlying evolutionary costs but also the transient provisioning costs could help to explain how and why parental care patterns evolved and exist in this amazing diversity. This thesis provides further understanding concerning consequences of evolutionary and provision cost in parental care thus brings clarification in possible factors shaping the evolution of parental care patterns.

Furthermore, this thesis provides a first insight in the analysis of buccal microbiota in mouthbrooding cichlids. While the significance of the gut and the skin microbiota is currently being investigated in regards to effects on the immune system, In order to manipulate fish health, other mucosal surfaces in vertebrates have largely been ignored. Direct and indirect benefits of the buccal microbiota and its development during mouthbrooding still have to be explored further.

The buccal microbial composition could be an adaptation to mouthbrooding mode and with its impact on the immune system and offspring development be assigned as an important parental effect. Parental buccal mucosa could be attributed to trans generational plasticity, just as the microbiota in the paternal brood pouch of syngnathids (Beemelmanns & Roth, in preparation), if upon mouth opening, this buccal mucosa is transferred to the offspring, and initial microbial gut colonisation takes place with parental bacteria (i.e. vertical transmission of commensal bacteria).

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass die vorliegende Dissertation mit dem Titel:

Parental investment and sexual immune dimorphism in cichlids and syngnathids

selbständig, mit der Beratung meiner Betreuer, von mir verfasst wurde.

Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein bisher erstes und einziges Promotionsverfahren.

Kapitel 1 und Kapitel 3 wurden zur Begutachtung in Fachzeitschriften eingereicht. Die Koautoren aller Kapitel befinden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Anteil der Autoren an den Manuskripten wird im Abschnitt „Author contributions“ erläutert.

Kiel, den 21.03.2017

Isabel Salome Keller

AUTHOR CONTRIBUTIONS

Chapter I:

IS, OR and WS conducted fieldwork and collected samples. **IS** and OR conducted the laboratory work. Data were collected and analysed by **IS**, TB & SK. OR and TBHR contributed materials and analysis tools. **IS**, OR and WS wrote and edited the manuscript.

Chapter II:

OR designed the experiment. **IS** and MH collected samples. **IS** and MH conducted laboratory work. Data was collected and analysed by **IS**. OR and TBHR contributed materials and analysis tools. **IS** and OR wrote and edited the manuscript.

Chapter III:

IS and OR designed the experiment. **IS** performed the experiment and conducted laboratory work. **IS** collected and analysed data. OR and TBHR contributed materials and analysis tools. **IS**, OR and WS wrote and edited the manuscript.

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